IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Nielsen *et al.*	Confirmation No.: 5726
Serial No.: 10/612,665	Art Unit: 1649
Filed: July 1, 2003	Examiner: Dutt, Aditi
For: RECOMBINANT TISSUE PROTECTIVE CYTOKINES AND ENCODING NUCLEIC ACIDS THEREOF FOR THE PROTECTION, RESTORATION AND ENHANCEMENT OF RESPONSIVE CELLS, TISSUES AND ORGANS	Attorney Docket No: 10165-022-999

DECLARATION OF MICHAEL L. BRINES, M.D., PH.D.

Sir:

I, MICHAEL L. BRINES, do hereby declare and state:

1. I am an inventor of the invention described and claimed in the above-identified patent application (hereinafter the "'665 application"). I am presently Chief Scientific Officer at Warren Pharmaceuticals, Inc., licensee of the '665 application.

2. I have over thirty years of experience in biological research and clinical investigation. I am a certified member of the American Board of Internal Medicine. My academic and technical experience and honors, and a list of my publications, are set forth in my curriculum vitae, a copy of which is attached hereto as Appendix A.

3. I have read and am familiar with the '665 application, the pending claims and the outstanding Office Action. I understand that the technology of the '665 application relates to the use of recombinant erythropoietin ("EPO") muteins (referred to herein as "muteins") for protecting, maintaining, or enhancing the viability of responsive cells, tissues, and organs isolated from the mammalian body and for protecting against or preventing tissue injury, or restoring or rejuvenating tissue or tissue function in a mammal.[1]

[1] For ease of reference, I will use the term "tissue protection" instead of the phrase "protecting, maintaining, or enhancing the viability of responsive cells, tissues, and organs isolated from the mammalian body and for protecting against or preventing tissue injury, or restoring or rejuvenating tissue or tissue function in a mammal." Likewise, the ability of a mutein to protect, maintain, or enhance the viability of responsive

These muteins have amino acid changes that reduce their erythropoietic activity compared to that of wild-type recombinant EPO, but which retain their tissue protective activity. I have been informed and believe that the claims of the '665 application are subject to a rejection based on the contention that the specification of the '665 application does not enable a person skilled in the art to make and use the claimed methods.

4. In the following paragraphs I will present evidence that shows that EPO muteins with reduced erythropoietic activity can provide tissue protective activity in all tissues that coexpress the EPO receptor ("EpoR") and the common β receptor ("βcR"). I present an analysis of the data in the '665 application and supplemental experimental data, citing a non-exhaustive list of examples from the literature, that demonstrate that EPO muteins and other tissue protective EPOs have tissue protective effects in a wide range of different tissues.

I. Reduced Erythropoietic Activity of EPO muteins

5. The '665 application discloses muteins that have reduced erythropoietic activity, *i.e.*, are deficient for signaling through the EpoR homodimer (the "Classical EPO Receptor"). The '665 application provides the results of experiments demonstrating that the muteins S100E, R103E, and R150E have reduced erythropoietic activity. See '665 application at p. 126, l. 12 to p. 128, l. 13. Moreover, as of July 2002, using the disclosure of the '665 application and routine experimentation, any EPO mutein could be generated and tested for whether it has reduced erythropoietic activity *in vitro* and *in vivo*. For example, the Satake paper cited in the '665 application at p. 42, ll. 10-11 discloses widely used assays for determining whether or not a form of EPO is erythropoietic *in vivo* (Satake, 1990, *Biochimica et Biophysica Acta* 1038:125-29; all references cited herein are provided in Appendix E).

6. We have also subsequently found that a mutein with two substitutions, K45D/S100E, is significantly reduced for erythropoietic activity. See Appendix B.

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cells, tissues, and organs isolated from the mammalian body or to protect against or prevent tissue injury, or restore or rejuvenate tissue or tissue function will be referred to as its "tissue protective" activity.

II. Tissue Protective Activity of EPO Muteins

7. The '665 application provides the results of experiments that demonstrate that EPOs with mutations in each of the four domains required for signaling through the Classical EPO Receptor have reduced erythropoietic activity yet maintain their tissue protective functionality in *in vitro* assays and *in vivo* in various tissues, including tissues of the central nervous system and peripheral nervous system.

8. The '665 application provides results of experiments that demonstrate that the EPO muteins S100E, R103E and R150E are tissue protective in well-established *in vitro* assays for apoptosis.

9. These results were confirmed and extended in experiments conducted subsequent to the '665 application. For example, the mutein R150E was found to prevent P19 cell apoptosis (see Appendix B). In addition, Leist *et al.* found that S100E and R103E reduce NMDA-induced apoptosis of rat hippocampal neurons (2004, *Science* 305:239-242; "Leist").

10. The ability of a mutein to confer tissue protection *in vitro* correlates with its tissue protective function *in vivo*. For example, the '665 application discloses that EPO muteins are tissue protective several *in vivo* assays for tissue injury. For instance, the '665 application shows that the S100E mutein protects the function of the injured spinal cord in an animal model, and the EPO muteins S100E, R103E, R150E, and K45D/S100E are tissue protective in retina.

11. Numerous studies have corroborated and extended our discovery that EPO muteins are tissue protective. For example, Villa *et al.* demonstrates the ability of S100E to improve neurological function after stroke (2007, *Journal of Cerebral Blood Flow & Metabolism* 27:255-63; "Villa"). Villa found that S100E administered three hours after ischemia significantly improved the performance of experimental rats in two tests for sensorimotor functions.

12. Moreover, in studies conducted by Applicants subsequent to the filing date of the '665 application, the EPO muteins S100E, R103E, and K45D/S100E were found to be tissue protective in assays for motor function following compression injury of the

sciatic nerve in rats. See Appendix B. Based on these studies, it is clear that EPO muteins protect central nervous system tissue and peripheral nervous system tissue.

13. It is also noted that similar tissue protective results were obtained with chemically-modified, nonerythropoietic forms of EPO, in which the charge of the amino acid side chains is altered by chemical modifications. This is significant because chemical modification of amino acids in EPO domains required for binding to the Classical EPO Receptor demonstrates the importance of these positions for binding to the Classical EPO Receptor. Thus, the EPO is reduced for its erythropoietic activity, but its tissue protective function is unimpaired.

14. For example, Villa demonstrated that carbamylated EPO (EPO in which the lysines are modified by the addition of carbamoyl moieties; "CEPO") protects against ischemic damage and improves post-ischemic neurological function as evaluated by motor function. See Villa. Leist demonstrated the neuroprotective effect of CEPO by showing that it reduced NMDA-induced apoptosis in cultured hippocampal cells. Leist confirmed these *in vitro* results with *in vivo* studies that demonstrated that CEPO is tissue protective in a rat stroke model, in a spinal cord injury model, in a multiple sclerosis model, and in a diabetic neuropathy model. See Leist.

15. Additional studies published after the '665 application's filing date demonstrate that CEPO is tissue protective in a wide range of tissues and injuries. Fiordaliso *et al.* showed that CEPO prevents apoptosis in cardiomyocytes in cell culture (2005, *PNAS* 102:2046-2051; "Fiordaliso"). As predicted by these *in vitro* data, CEPO was found to be cardioprotective during ischemia in an *in vivo* model of myocardial infarction. Moon *et al.* demonstrated that CEPO protects cardiac tissue from toxin-induced stress and oxidative stress (2005, *J. Pharmacol. Exp. Therapeutics* 316:999-1005; "Moon"). Moon showed the anti-apoptotic effect of CEPO on isolated cardiomyocytes and in the heart tissue of a rat model of myocardial ischemia. Imamura *et al.* demonstrated that CEPO protects kidneys in from reperfusion injury following ischemia in a rat model, demonstrating the anti-apoptotic activity of CEPO in kidney tissue (2007, *Biochem Biophys Res Comm*, 353:786-792; "Imamura"). Similarly, Erbayraktar and colleagues demonstrated the neuroprotective activity of CEPO in an animal model of radiation-induced necrosis of tissue. See Erbayraktar, *et al.*, 2006, *Molecular Medicine* 12:74-80. Schmidt *et al.* showed that CEPO

protected neurons in an animal model of diabetes from diabetes-induced neuritic dystrophy (2008, *Exp. Neurol.* 209:161-170). Bianchi *et al.* demonstrated that CEPO preserved the function of neurons in the peripheral nervous system after exposure to a neurotoxic drug (2006, *Clin. Cancer Res.* 12(8):2607-2612). Mahmood *et al.* demonstrated that administration of CEPO improved spatial learning in the Morris water maze assay following traumatic brain injury (2007, *J. Neurosurg.* 107:392-397). Finally, Mennini *et al.* demonstrated that CEPO can restore the function of the nervous system by demonstrating that CEPO significantly improves the behavioral scores of wobbler mice (2006, *Molecular Medicine* 12:153-160).

16. We also found that other chemically-modified forms of EPO are nonerythropoietic yet maintain their tissue protective activity, based on our observations using the *in vivo* sciatic nerve compression assay. For instance, EPOs with modified lysines (modified by not only carbamylation but also carboxymethylation, PEGylation, or succinylation), modified arginines (by cyclohexanedionation), tyrosines (by trinitrophenylation), and an EPO in which the disulfide bonds are reduced by iodoacetamidation were all tissue protective in this assay. Moreover, in an animal model of cerebral ischemia, EPOs with carbamylated, carboxymethylated, PEGylated, and PEGylated/carbamylated lysines, an EPO in which the arginines are modified by phenylglyoxalation, EPO with trinitrophenylated tyrosines, and an EPO with disulfides reduced by iodoacetamidation were each also found to be tissue protective. See Appendix C.

17. These data show that EPOs having amino acids modified such that their erythropoietic activity is reduced protect tissues as diverse as heart tissue, kidney tissue, endothelial tissue, and central nervous system tissue. Similarly, these modified EPOs protect tissues from ischemias, such as stroke and infarction, toxin-induced stress, oxidative stress, reperfusion injury, NMDA-induced apoptosis, spinal cord compression injury, sciatic nerve compression injury, and radiation-induced necrosis. Further, the beneficial effect of modified EPOs on tissue function has been demonstrated by evaluating motor function, spatial learning, and other behavioral parameters.

18. In addition to the above experiments that demonstrate the tissue protective capabilities of EPO muteins, the '665 application also provides *in vitro* and *in vivo* assays to test the tissue protective activity of any EPO mutein. These assays are routine

assays, which were well-accepted as models for human disease as of July 2002. Because these assays are predictive of tissue protective activity in general, and because we discovered that EPO can cross tight endothelial cell barriers, positive results in these assays can be extrapolated to all EPO-responsive tissues and organs and to the diseases and disorders that result from the dysfunction of these tissues and organs.

19. The '665 application describes, for example, the use of the well-established P19 neural cell assay to test the tissue-protective activity of a compound *in vitro*. Upon withdrawal of serum, P19 cells undergo apoptosis. The ability of a compound to prevent serum-deprivation–induced cell death in this assay demonstrates the cell protective capacity of that compound. See also Ho *et al.*, 1998, *J. Neuroimmunol.* 89:142-149; "Ho." Similar assays for other cell types were well-known in the art. For example, NMDA-induced apoptosis in hippocampal neurons provides such an assay system. See, *e.g.*, Prehn (1994, *PNAS* 91:12599-12603). An assay for determining the effect of oxidative stress in cardiomyocytes was taught in Zorov (2000, *J. Exp. Med.* 192:1001-1014).

20. These *in vitro* assays simulate pathological conditions *in vivo*, and the results obtained in such *in vitro* systems are predictive of results *in vivo*. See also, *e.g.*, Ho, p. 147, 1st paragraph of "Discussion." This correlation between results in *in vitro* and *in vivo* tests is also demonstrated by the experiments presented in the '665 application. The ability of wild-type, chemically-modified, and mutein EPOs to confer protection on cells in culture was validated in several *in vivo* models of tissue protection (see the Examples section of the '665 application and the data discussed in paragraphs 10-16 above).

21. Examples of routine *in vivo* and *ex vivo* assays using animal model systems are also described in the '665 application. For instance, the *ex vivo* assay described in Example 7 (pp. 112-113) may be used to assess a particular mutein for its ability to maintain the function of a heart prepared for transplantation. The *in vivo* assay described in Example 8 (p. 113) may be used to assess a particular mutein for its ability to protect heart function during an ischemic event. The middle cerebral artery occlusion assay can be used to assess the neuroprotective effect of a mutein following brain ischemia (pp. 118-120), or assays that test a mutein's protection against spinal cord injury (pp. 115-118) or damage due to retinal ischemia (p. 114 and pp. 128-129) may be used. Other *in vivo* assays provided in the '665 application include assays for inflammation (pp. 120-121), neuroprotection as

measured by neuronal trauma induced by water intoxication (pp. 111-112), an assay for restoration of cognitive function following brain injury (pp. 114-115), and the kainate model for assessing neurotoxic injury (p. 115). Using the descriptions of these assays in the '665 application, any mutein could be tested for the protection of various types of tissue from injury.

22. Additional assays useful to a biomedical scientist for assessing the tissue protective effects of a compound in different tissues were well-known in July 2002. For example, the tissue protective effect of a compound on kidney cells could be tested in a model system for ischemia/reperfusion injury (*e.g.*, Ysebaert *et al.*, 2000, *Nephrol. Dial. Transplant* 15:1562-74). Assays for evaluating the protective effect of a compound on heart tissue *in vivo* and *in vitro* were well-established in the art. See, *e.g.*, Kajstura *et al.*, 2000, *Diabetes* 50:1414-1424. Lack of oxygen is the cause of tissue damage in many different pathological conditions. Such ischemic events could be simulated by occlusion of arteries in animal models. For example, the middle cerebral artery occlusion model was known to recapitulate damage caused by stroke (see '665 application and Brines *et al.*, 2000, *PNAS* 97:10526-10531; "MCA occlusion" in Materials and Methods). Ischemic damage could be quantified by measuring the extent of the tissue injury, *e.g.*, the size of the lesion, and by measuring functional parameters, *e.g.*, learning and memory, motor coordination, *etc.* Another well-established animal model system for testing the effects of a compound on the function of the nervous system is the "wobbler mouse" (Duchen *et al.*, 1968, *J. Neurol. Neurosurg. Psychiat.* 31:535-542). Thus, equipped with the teachings of the '665 application and merely routine experimentation, it was possible to identify and test EPO muteins with reduced erythropoietic activity suitable for tissue protection.

III. The Tissue Protective and Erythropoietic Activities of EPO are Mediated by Separate and Distinct Pathways

23. The evidence presented above shows that EPOs that are unable to bind the Classical EPO Receptor are effective at conferring tissue protection. We discovered that this is because EPO provides tissue protective activity via a pathway distinct from the pathway it uses to exert its erythropoietic effects (Brines *et al.*, 2004, *PNAS* 101:14907-14912; "Brines 2004"). Rather than conferring tissue protection through Classical EPO Receptor binding, EPO's tissue protective activity is mediated through its interaction with a different receptor, a heteromer of EpoR and βcR (the "Tissue Protective Receptor Complex").

βcR (also known as CD131) is the signal-transducing subunit shared by the granulocyte-macrophage colony stimulating factor, IL-3 receptor, and IL-5 receptor (Brines 2004).

24. EPO signaling through the Classical EPO Receptor (present predominantly on blood forming cells) elicits an increase in erythrocytes, platelets, and blood pressure. Activation by EPO of the Tissue Protective Receptor Complex (present in most tissues) leads to a wider range of tissue protective effects. This is because EPO signaling through the Tissue Protective Receptor Complex serves a basic molecular function common to many tissues. For instance, we know that EPO protects against tissue damage caused by the general proinflammatory cytokine TNFα, reduces the extent of TNFα-induced damage, and promotes healing and regeneration of affected tissues. We also know that EPO prevents oxidative stress–induced damage to cells and tissues.

25. It is well-recognized that various diseases impact the function and viability of cells, tissues and organs through these common pathways, which EPO is able to antagonize. In the case of neurodegeneration, for example, it has been noted that, irrespective of the primary causes of individual neurodegenerative diseases, the onset of oxidative stress resulting from free radical mechanisms causes neuronal cell death and progression of the disease. See Jenner, 1996, *Pathol. Biol.* (Paris), 44(1):57-64, Abstract.

26. Based on our discovery that peripherally-administered EPO can cross tight endothelial cell barriers, such as the blood-brain barrier and blood-retinal barrier, exogenously administered EPO is capable of conferring tissue protection on any tissue that expresses the Tissue Protective Receptor Complex, whether or not it is surrounded by an endothelial barrier.

27. Therefore, as a tissue protective cytokine, EPO counteracts the tissue damage at the root of the variety of diseases and disorders disclosed in the '665 application and recited in the claims. EPO's ability to prevent the cell death and tissue damage that is common to many disease conditions demonstrates the widespread therapeutic implications of EPO and EPO muteins, regardless of the initial cause of the condition or its other symptoms.

28. Thus, an EPO mutein found to be tissue protective in one or more of the assays described in ¶¶ 8-22 above can be expected to exert tissue protective effects in any

tissue that coexpresses EpoR and βcR. Appendix D contains a non-exhaustive list of references that describe EpoR-expressing cells, tissues, and organs. Cell types demonstrated to express EpoR include, but are not limited to, neurons and glial cells, astrocytes, endothelial cells, myocardiocytes, macrophages, retinal cells, cells of the adrenal cortex and medulla, small bowel, spleen, liver, kidney and lung. Appendix D also provides a list of the many tissues that express the βcR (see reference 18, attached at the end of Appendix D). Thus, I believe that the tissue protective activity of EPO, which is mediated through the Tissue Protective Receptor Complex, may be found in all tissues that express EpoR and βcR.

IV. CONCLUSION

29. In summary, I have presented data that demonstrate that EPO muteins have tissue protective activity in many types of diseases and tissue injuries, citing a non-exhaustive list of examples from the '665 application, the scientific literature, and from Applicants' own studies conducted subsequent to the filing date of the '665 application.

30. I declare further that all statements made in this Declaration of my own knowledge are true, that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

June 29, 2008
Date

Michael L. Brines, M.D., Ph.D.

Attachments:

Appendix A: Curriculum Vitae of Dr. Michael L. Brines, M.D., Ph.D.
Appendix B: Examples of nonerythropoietic, tissue protective EPO muteins
Appendix C: Examples of chemical modification that produce nonerythropoietic, tissue protective erythropoietins
Appendix D: References on tissues that express EPO receptors: EpoR and βcR
Appendix E: Cited References